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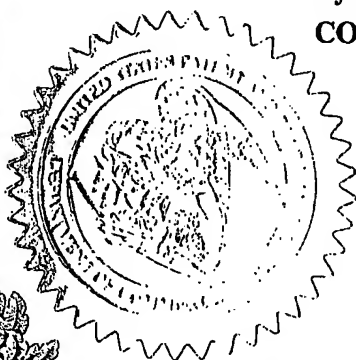
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# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(b)(2).

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<input checked="" type="checkbox"/> Additional inventors are being named on <u>1</u> separately numbered sheets attached hereto.					
TITLE OF THE INVENTION (280 characters max)					
TUBERCULOSIS VACCINE WITH IMPROVED EFFICACY					
CORRESPONDENCE ADDRESS					
<input checked="" type="checkbox"/> Customer Number: <b>6449</b>					
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<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27			Filing Fee Amount: \$160.00		
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted,

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APPLICATION DATA SHEET

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## Tuberculosis Vaccine with Improved Efficacy

### Specification

5

The present invention relates to novel recombinant vaccines providing protective immunity especially against tuberculosis.

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Tuberculosis (TB) caused by *Mycobacterium tuberculosis* remains a significant global problem. It is estimated that one third of the world's population is infected with *M.tuberculosis* (Kochl, 1991). In many countries the only measure for TB control has been vaccination with *M.bovis* bacille Calmette-Guérin (BCG). The overall vaccine efficacy of BCG against TB, however, is about 50 % with extreme variations ranging from 0 % to 80 % between different field trials (Roche et al., 1995). Thus, BCG should be improved, e.g. by genetic engineering, to provide a vaccine for better TB control (Murray et al., 1996; Hess and Kaufmann, 1993). The widespread emergence of multiple drug-resistant *M.tuberculosis* strains additionally underlines the urgent requirement for novel TB vaccines (Grange, 1996).

20

*M.tuberculosis* belongs to the group of intracellular bacteria that replicate within the phagosomal vacuoles of resting macrophages, thus protection against TB depends on T cell-mediated immunity (Kaufmann, 1993). Several studies in mice and humans, however, have shown that *Mycobacteria* stimulate antigen-specific, major histocompatibility complex (MHC) class II- or class I-restricted CD4 and CD8 T cells, respectively (Kaufmann, 1993).

25

The important role of MHC class I-restricted CD8 T cells was convincingly demonstrated by the failure of  $\beta 2$ -microglobulin ( $\beta 2m$ ) deficient mice to control experimental *M.tuberculosis* infection (Flynn et al., 1993). Because these mutant mice lack MHC class I, functional CD8 T cells cannot

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develop. In contrast to *M.tuberculosis* infection,  $\beta 2m$ -deficient mice are capable of controlling certain infectious doses of the BCG vaccine strain (Flynn et al., 1993; Ladel et al., 1995). Furthermore, BCG vaccination of  $\beta 2m$ -deficient mice prolonged survival after subsequent *M.tuberculosis* infection whereas BCG-immunized C57BL/6 resisted TB (Flynn et al., 1993). This differential CD8 T cell dependency between *M.tuberculosis* and BCG may be explained as follows: *M.tuberculosis* antigens gain better access to the cytoplasm than antigens from BCG leading to more pronounced MHC class I presentation (Hess and Kaufmann, 1993). Consequently, a more effective CD8 T cell response is generated by *M.tuberculosis*. This notion was recently supported by increased MHC class I presentation of an irrelevant antigen, ovalbumin, by simultaneous *M.tuberculosis*, rather than BCG, infection of antigen presenting cells (APC) (Mazzaccaro et al., 1996).

Secreted proteins of *M.tuberculosis* comprise a valuable source of antigens for MHC class I presentation. Recently, a DNA vaccine encoding the secreted antigen Ag85A elicited MHC class I-restricted CD8 T cell responses in mice which may contribute to defence against TB (Huygen et al., 1996). In general, evidence is accumulating that immunization with secreted protein antigens of *M.tuberculosis* induce some protection against TB in guinea pigs and mice (Horwitz et al., 1995; Andersen, 1994). An important goal towards the development of improved TB vaccines based on BCG, therefore, is to augment the accessibility of secreted BCG-specific antigens to the cytoplasm of infected APC. Subsequent delivery of peptides derived from these secreted proteins into the MHC class I presentation pathway may potentiate the already existing BCG-specific immune response for preventing TB.

The phagolysosomal escape of *L.monocytogenes* represents a unique mechanism to facilitate MHC class I antigen presentation of listerial antigens (Berche et al., 1987; Portnoy et al., 1988). Listeriolysin (Hly), a

pore-forming sulfhydryl-activated cytolysin, is essential for the release of *L.monocytogenes* microorganisms from phagolysosomal vacuoles into the cytosol of host cells (Gaillard et al., 1987; Portnoy et al., 1988). This escape function was recently transferred to *Bacillus subtilis* and to  
 5 attenuated *Salmonella* ssp. strains (Bielecki et al., 1991; Gentshev et al., 1995; Hess and Kaufmann, 1997). Hly expression by an asporogenic *B.subtilis* mutant strain or in *Salmonella* ssp. results in bacterial escape from the phagolysosome into the cytosol of J774 macrophage-like cells (Bielecki et al., 1991; Gentshev et al., 1995; Hess and Kaufmann, 1997).

10 WO 99/101496 and Hess et al. (1998) disclose recombinant *Mycobacterium bovis* strains that secrete biologically active Listeriolysin fusion proteins. These *M.bovis* strains have been shown to be effective vaccines against TB in several animal models.

15 According to the present invention Hly was expressed in urease-deficient BCG strains. These urease-deficient BCG strains exhibit an increased Hly activity in phagosomes and in turn improved pore formation in the endosomal membranes leading to superior immunoprotectivity.

20 Thus, a first aspect of the present invention is a bacterial cell, particularly a *Mycobacterium* cell which is urease-deficient and comprises a recombinant nucleic acid molecule encoding a fusion polypeptide comprising (a) at least one domain from a polypeptide, wherein said  
 25 polypeptide domain is capable of eliciting an immune response in a mammal, and (b) a phagolysosomal escape domain. It is preferred that the cell is capable of expressing the nucleic acid molecule of the invention. More preferably, the cell is capable of secreting the fusion polypeptide and/or of providing it in a form suitable for MHC class I-restricted antigen  
 30 recognition.

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The bacterial cell of the invention is a urease-deficient cell, e.g. a gram-negative or a gram-positive bacterial cell, preferably a *Mycobacterium* cell. The urease-deficiency may be achieved by partially or completely inactivating one or several cellular nucleic acid molecules which code for  
 5 a urease subunit, particularly *ureA* encoding for urease subunit A, *ureB* coding for urease subunit B and/or *ureC* coding for urease subunit C. The sequences of *ureA*, *ureB* and *ureC* in *Mycobacteria*, particularly *M.bovis* and *M.tuberculosis* and the proteins encoded thereby are described by Reyrat et al. (1995) and Clemens et al. (1995), which are incorporated  
 10 herein by reference.

Preferably the urease-deficient bacterial strain is obtained by deletions and/or insertions of one or several nucleotides in urease subunit - coding nucleic acid sequences and/or their expression control sequences.  
 15 Deletions and/or insertions may be generated by homologous recombination, transposon insertion or other suitable methods.

In an especially preferred embodiment the *ureC* sequence is inactivated, e.g. by constructing a suicide vector containing a *ureC* gene disrupted by  
 20 a selection marker gene, transforming the target cell with the vector and screening for selection marker-positive cells having a urease negative phenotype as described by Reyrat et al. (1995).

The cell of the invention is preferably an *M.bovis* cell, a *M.tuberculosis* cell,  
 25 particularly an attenuated *M.tuberculosis* cell or other *Mycobacteria*, e.g. *M.microti*, *M.smegmatis*, *M.canettii*, *M.marinum* or *M.fortuitum* or *Mycobacteria* as described by Reyrat et al. (1995).

The *Mycobacterium* cell of the invention comprises a recombinant nucleic acid molecule, e.g. the nucleic acid molecule in SEQ ID No.1. This nucleic acid molecule comprises a signal peptide coding sequence (nucleotide 1 -  
 30 120), a sequence coding for an immunogenic domain (nucleotide 121 -

153), a peptide linker coding sequence (nucleotide 154 - 210), a sequence coding for a phagolysosomal domain (nucleotide 211 - 1722), a further peptide linker coding sequence (nucleotide 1723 - 1800) and a sequence coding for a random peptide (nucleotide 1801 - 1870). The corresponding amino acid sequence is shown in SEQ ID No.2.

The nucleic acid contains at least one immunogenic domain from a polypeptide. The immunogenic domain may be derived from an organism of the genus *Mycobacterium*, preferably from *Mycobacterium tuberculosis* or from *Mycobacterium bovis*. This domain has a length of at least 6, preferably of at least 8 amino acids. The immunogenic domain is preferably a portion of a native *Mycobacterium* polypeptide. However, within the scope of the present invention is also a modified immunogenic domain, which is derived from a native immunogenic domain by substituting, deleting and/or adding one or several amino acids.

The immunogenic domain is however not restricted to *Mycobacterium* antigens and can be selected from autoantigens, tumor antigens and pathogen antigens such as virus antigens, parasite antigens, bacterial antigens in general and immunogenic fragments thereof. Specific examples for suitable tumor antigens are human tumor antigens such as the p53 tumor suppressor gene product (Houbiers et al., 1993) and melanocyte differentiation antigens, e.g. Melan-A/MART-1 and gp100 (van Elsas et al., 1996). Specific examples for suitable virus antigens are human tumor virus antigens such as human papilloma virus antigens, e.g. antigens E6 and E7 (Bosch et al., 1991), influenza virus antigens, e.g. influenza virus nucleoprotein (Matsui et al., 1995; Fu et al., 1997) or retroviral antigens such as HIV antigens, e.g. the HIV-1 antigens p17, p24, RT and Env (Harrer et al., 1996; Haas et al., 1996). Specific examples for suitable parasite antigens are *Plasmodium* antigens such as liver stage antigen (LSA-1), circumsporozoite protein (CS or allelic variants cp26 or cp29), thrombospondin related anonymous protein (TRAP), sporozoite threonine

and asparagine rich protein (STARP) from *Plasmodium falciparum* (Aidoo et al., 1995) and *Toxoplasma* antigens such as p30 from *Toxoplasma gondii* (Khan et al., 1991; Bulow and Boothroyd, 1991). Specific examples for suitable bacterial antigens are *Legionella* antigens such as Major secretory protein from *Legionella pneumophila* (Blander and Horwitz, 1991).

The immunogenic domain is capable of eliciting an immune response in a mammal. This immune response can be a B cell-mediated immune response. Preferably, however, the immunogenic domain is capable of eliciting a T cell-mediated immune response, more preferably a MHC class I-restricted CD8 T cell response.

The domain capable of eliciting an immune response is more preferably selected from immunogenic peptides or polypeptides from *M.bovis* or *M.tuberculosis* or from immunogenic fragments thereof. Specific examples for suitable antigens are Ag85B (p30) from *M.tuberculosis* (Harth et al., 1996), Ag85B ( $\alpha$ -antigen) from *M.bovis* BCG (Matsuo et al., 1988), Ag85A from *M.tuberculosis* (Huygen et al., 1996) and ESAT-6 from *M.tuberculosis* (Sorensen et al., 1996, Harboe et al., 1996 and Andersen et al., 1995). More preferably, the immunogenic domain is derived from the antigen Ag85B. Most preferably, the immunogenic domain comprises the sequence from aa,41 to aa.51 in SEQ ID No.2.

The recombinant nucleic acid molecule according to the present invention further comprises a phagolysosomal escape domain, i.e. a polypeptide domain which provides for an escape of the fusion polypeptide from the phagolysosome into the cytosol of mammalian cells. Preferably, the phagolysosomal escape domain is a *Listeria* phagolysosomal escape domain, which is described in US 5,733,151, herein incorporated by reference. More preferably, the phagolysosomal escape domain is derived from the organism *L.monocytogenes*. Most preferably, the phagolysosomal domain is encoded by a nucleic acid molecule selected from: (a) a

nucleotide sequence comprising nucleotides 211 - 1722 as shown in SEQ ID No.1, (b) a nucleotide sequence which encodes for the same amino acid sequence as the sequence from (a), and (c) a nucleotide sequence hybridizing under stringent conditions with the sequence from (a) or (b).

5

Apart from the nucleotide sequence depicted in SEQ ID No.1 the present invention also comprises nucleic acid sequences hybridizing therewith. In the present invention the term "hybridization" is used as defined in Sambrook et al. (Molecular Cloning. A laboratory manual, Cold Spring Harbor Laboratory Press (1989), 1.101-1.104). In accordance with the present invention the term "hybridization" is used if a positive hybridization signal can still be observed after washing for one hour with 1 X SSC and 0.1 % SDS at 55°C, preferably at 62° C and more preferably at 68°C, particularly for 1 hour in 0.2 X SSC and 0.1 % SDS at 55°C, preferably at 62°C and more preferably at 68°C. A sequence hybridizing with a nucleotide sequence as per SEQ ID No.1 under such washing conditions is a phagolysosomal escape domain encoding nucleotide sequence preferred by the subject invention.

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A nucleotide sequence encoding a phagolysosomal escape domain as described above may be directly obtained from a Listeria organism or from any recombinant source e.g. a recombinant E.coli cell containing the corresponding Listeria nucleic acid molecule or a variant thereof as described above.

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Preferably, the recombinant nucleic acid molecule encoding for a fusion polypeptide contains a signal peptide encoding sequence. More preferably, the signal sequence is a signal sequence active in Mycobacteria, preferably in M.bovis, e.g. a native M.bovis signal sequence. A preferred example of a suitable signal sequence is the nucleotide sequence coding for the Ag85B signal peptide which is depicted in SEQ ID No.1 from nucleotide 1 to 120.

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Further, it is preferred that a peptide linker be provided between the immunogenic domain and the phagolysosomal escape domain. Preferably, said peptide linker has a length of from 5 to 50 amino acids. More preferably, a sequence encoding a linker as shown in SEQ ID No.1 from  
5 nucleotide 154 to 210 or a sequence corresponding thereto as regards the degeneration of the genetic code.

The nucleic acid may be located on a recombinant vector. Preferably, the recombinant vector is a prokaryotic vector, i.e. a vector containing  
10 elements for replication or/and genomic integration in prokaryotic cells. Preferably, the recombinant vector carries the nucleic acid molecule of the present invention operatively linked with an expression control sequence. The expression control sequence is preferably an expression control  
15 sequence active in Mycobacteria, particularly in M.bovis. The vector can be an extrachromosomal vector or a vector suitable for integration into the chromosome. Examples of such vectors are known to the man skilled in the art and, for instance, given in Sambrook et al. supra.

In a further aspect of the present invention a urease-deficient bacterial cell  
20 e.g., a Mycobacterium cell, preferably an M.bovis cell is provided which comprises at least one nucleic acid molecule encoding a phagolysosomal escape peptide or polypeptide. Even if the phagolysosomal escape peptide or polypeptide is not fused with an antigen, a surprising improvement of the immunogenic properties is found.

25 The recombinant bacterial cell which is provided according to this further aspect of the present invention may contain at least one further recombinant, e.g. heterologous nucleic acid molecule encoding a peptide or polypeptide capable of eliciting an immune response in a mammal. Said  
30 further immunogenic peptide or polypeptide may be selected from Mycobacterium antigens or, in a wider sense, from autoantigens, tumor antigens, pathogen antigens and immunogenic fragments thereof. The

nucleic acid molecule coding for the further peptide or polypeptide may be situated on the same vector as the fusion gene. However, it may, for example, also be situated on a different plasmid, independently of the fusion gene; or be chromosomally integrated.

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Surprisingly, it was found that a Mycobacterium cell according to the present invention has an intracellular persistence in infected cells, e.g. macrophages, which is equal or less than the intracellular persistence of a corresponding native Mycobacterium cell which does not contain the recombinant nucleic acid molecule.

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The present invention also refers to a pharmaceutical composition comprising as an active agent a cell as defined above, optionally together with pharmaceutically acceptable diluents, carriers and adjuvants.

15

Preferably, the composition is a living vaccine suitable for administration to a mammal, preferably a human. The actually chosen vaccination route depends on the choice of the vaccination vector. Administration may be achieved in a single dose or repeated at intervals. The appropriate dosage depends on various parameters such as the vaccinal vector itself or the route of administration. Administration to a mucosal surface (e.g. ocular, intranasal, oral, gastric, intestinal, rectal, vaginal or urinary tract) or via the parenteral route (e.g. subcutaneous, intradermal, intramuscular, intravenous or intraperitoneal) might be chosen.

20

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Further, the present invention pertains to a method for preparing a recombinant bacterial cell as defined above. According to the first aspect, this method comprises the steps of (i) providing a urease-deficient bacterial cell, particularly a Mycobacterium cell, (ii) inserting a recombinant nucleic acid molecule into said bacterial cell, said nucleic acid molecule encoding a fusion polypeptide comprising (a) at least one domain from a polypeptide wherein said domain is capable of eliciting an immune response in a mammal and (b) a phagolysosomal escape domain, and (iii) cultivating the

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cell obtained according to step (ii) under suitable conditions. Preferably, a cell is obtained which is capable of expressing said nucleic acid molecule. More preferably, the cell is an *M.bovis* cell.

- 5 According to the further aspect, this method comprises the step of (i) providing an urease-deficient bacterial cell, particularly a *Mycobacterium* cell, (ii) inserting a recombinant nucleic acid molecule into said bacterial cell, said nucleic acid molecule encoding a phagolysosomal escape peptide or polypeptide, and (iii) cultivating the cell obtained according to (ii) under  
10 suitable conditions.

If desired, the method of the present invention comprises inserting at least one further recombinant nucleic acid molecule into the bacterial cell, said further recombinant nucleic acid molecule encoding a peptide or  
15 polypeptide capable of eliciting an immune response in a mammal.

Finally, the present invention relates to a method for the preparation of a living vaccine comprising formulating the recombinant cell in a pharmaceutically effective amount with pharmaceutically acceptable  
20 diluents, carriers and/or adjuvants.

The invention will be further illustrated by the following figures and sequence listings.

- 25 Fig.1: the protective capacity of  $\Delta$ ureC BCG Hly in the aerosol model of murine tuberculosis. BALB/c mice were immunized i.v. with  $1 \times 10^6$  CFU  $\Delta$ ureC BCG Hly or BCG "Pasteur". 120 days post vaccination animals were challenged with H37Rv (200 organism/lung) via aerosol. Bacterial load in infected  
30 organs (spleen and lung) was assessed 30, 60 and 90 days post challenge. Each bar represents 10 animals.

SEQ ID No.1: shows the nucleotide sequence of a nucleic acid molecule according to the present invention.

SEQ ID No.2: shows the corresponding amino acid sequence of the nucleic acid molecule of SEQ ID No.1.

### Example

#### 1. Inactivation of the urease activity of BCG delta ureC.

To obtain a urease-deficient mutant, Reytrat et al. constructed a suicide vector containing a ureC gene disrupted by a kanamycin marker (the aph gene). Two micrograms of this construct were linearized with Sac I and electroporated into M. bovis BCG. Kanamycine resistant transformants were screened for urease negative phenotype (cf. Reytrat et al., 1995).

#### 2. Construction of the mycobacterial E. coli shuttle expression vector pMV306:Hly.

To transfer the phagosomal escape function (mediated by Hly of L. monocytogenes EGD Sv 1/2a), to BCG Pasteur (1173 P<sub>3</sub>) delta ureC, an E. coli-mycobacterial shuttle vector was used. The integrative plasmid pMV306, a precursor of vector pMV361, allows stable chromosomal expression of Hly.

A pLH-1-derived 1.7-kb PstI DNA fragment coding for an hly-hlyA (E. coli pHly152-specific hemolysin A) ORF was inserted into PstI site of plasmid pAT261. This resulting gene fusion codes for the expression of secreted proteins directed to the supernatant by the BCG-specific Ag85B signal peptide. The construct was termed pAT261:Hly and its XbaI-Sall DNA expression cassette under

transcriptional control of the hsp60 mycobacterial promoter was subsequently used for insertion into the parental pMV306 vector resulting in the construct pMV306:Hly. The DNA sequence of the hly-specific insertion sites in both mycobacterial expression plasmids was analyzed. The mature Hly fusion protein putatively consists of 30 aa at the N terminus and 52 aa at the C-terminal part of the fusion that partially belong to HlyA of *E. coli*.

### 3. Protective capacity

The expression vector pMV306:Hly was transformed into an urease deficient BCG strain. The resultant strain was designated  $r \Delta$ ureC/BCG Hly. The protective capacity of this urease-deficient mycobacterial strain in a model of murine tuberculosis is shown in Figure 1.

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## Claims

1. A bacterial cell which is urease-deficient and which comprises a recombinant nucleic acid molecule encoding a fusion polypeptide comprising (a) at least one domain from a polypeptide, wherein said polypeptide domain is capable of eliciting an immune response in a mammal, and (b) a phagolysosomal escape domain.
2. The cell of claim 1, wherein at least one cellular urease subunit encoding nucleic acid sequence is inactivated.
3. The cell of claim 2 wherein at least the cellular urease C subunit-encoding sequence is inactivated.
4. The cell of claim 1, wherein said phagolysosomal escape domain is a *Listeria* phagolysosomal escape domain.
5. The cell of claim 1, wherein said phagolysosomal domain is encoded by a nucleic acid molecule selected from:
  - (a) a nucleotide sequence comprising nucleotide 211 - 1722 as shown in SEQ ID No.1;
  - (b) a nucleotide sequence which encodes for the same amino acid sequence as the sequence from (a), and
  - (c) a nucleotide sequence hybridizing under stringent conditions with the sequence from (a) or (b).
6. The cell of claim 1, wherein the domain capable of eliciting an immune response is a peptide or polypeptide capable of eliciting MHC class I-restricted CD8 T cell responses.

7. The cell of claim 1 wherein the domain capable of eliciting an immune response is from a Mycobacterium polypeptide.
8. The cell of claim 7, wherein the domain capable of eliciting an immune response is selected from the Mycobacterium antigens Ag85B (M.tuberculosis), Ag85B (M.bovis), Ag85A (M.tuberculosis) and ESAT-6 (M.tuberculosis) or an immunogenic fragment thereof.
9. The cell of claim 8, wherein the domain capable of eliciting an immune response is the antigen Ag85B or an immunogenic fragment thereof.
10. The cell of claim 1, wherein the fusion polypeptide is preceded by a signal peptide sequence.
11. The cell of claim 1, wherein a peptide linker is located between the immune response eliciting domain and the phagolysosomal domain.
12. The cell of claim 1, wherein said nucleic acid molecule is operatively linked with an expression control sequence.
13. The cell of claim 12, wherein said expression control sequence is active in said cell.
14. The cell of claim 1 wherein said nucleic acid molecule is located on a vector.
15. The cell of claim 1 which is a Mycobacterium cell.
16. The cell of claim 16 which is a Mycobacterium bovis cell.

17. A bacterial cell which is urease-deficient and which comprises at least one recombinant nucleic acid molecule encoding a phagolysosomal escape peptide or polypeptide.

18. The cell of claim 17, which comprises at least one further recombinant nucleic acid molecule encoding a peptide or polypeptide capable of eliciting an immune response in a mammal.

19. The cell of claim 18 which is a Mycobacterium cell.

20. The cell of claim 19 which is a Mycobacterium bovis cell.

21. The cell of claims 1 or 17, wherein the domain or peptide or polypeptide capable of eliciting an immune response is selected from autoantigens, tumor antigens, virus antigens, parasite antigens, bacterial antigens and immunogenic fragments thereof.

22. The cell of claims 1 or 17, which is capable of expressing said at least one recombinant nucleic acid molecule.

23. The cell of claim 22, which is capable of secreting a polypeptide encoded by said at least one nucleic acid molecule.

24. The cell of claims 1 or 23, which has an intracellular persistence in infected macrophages which is equal or less than the intracellular persistence of a native Mycobacterium cell.

25. A pharmaceutical composition comprising as an active agent a cell of claims 1 or 17, optionally together with pharmaceutically acceptable diluents, carriers and adjuvants.

26. The composition of claim 25, which is a living vaccine suitable for administration to a mucosal surface or via the parenteral route.
27. A method for the preparation of a living vaccine comprising  
5     formulating a cell of claims 1 or 17 in a pharmaceutically effective amount with pharmaceutically acceptable diluents, carriers and adjuvants.
28. A method for preparing a recombinant bacterial cell of claim 1.  
10     comprising the steps:
  - (i)     providing a urease-deficient bacterial cell;
  - (ii)    inserting a recombinant nucleic acid molecule into said bacterial cell, said nucleic acid molecule encoding a fusion polypeptide comprising (a) at least one domain from a  
15     polypeptide, wherein said domain is capable of eliciting an immune response in a mammal, and (b) a phagolysosomal escape domain, and
  - (iii)   cultivating the cell obtained according to (ii) under suitable conditions.
29. The method of claim 28, wherein said cell is a M.bovis cell.
30. A method for preparing a recombinant bacterial cell of claim 17  
25     comprising the steps:
  - (i)     providing a urease-deficient bacterial cell;
  - (ii)    inserting a recombinant nucleic acid molecule into said bacterial cell, said nucleic acid molecule encoding a phagolysosomal escape peptide or polypeptide and
  - (iii)   cultivating the cell obtained according to (ii) under suitable  
30     conditions.

- 26 -

31. The method of claim 30 comprising inserting at least one further recombinant nucleic acid molecule into the bacterial cell, said further recombinant nucleic acid molecule encoding a peptide or polypeptide capable of eliciting an immune response in a mammal.

5

32. The method of claim 28 or 30, wherein the domain or peptide or polypeptide capable of eliciting an immune response is selected from autoantigens, tumor antigens, virus antigens, parasite antigens, bacterial antigens and immunogenic fragments thereof.

10

- 27 -

**Abstract**

5 The present invention relates to novel recombinant vaccines providing protective immunity against tuberculosis. Further, the present invention refers to novel recombinant nucleic acid molecules, vectors containing said nucleic acid molecules, cells transformed with said nucleic acid molecules and polypeptides encoded by said nucleic acid molecules.

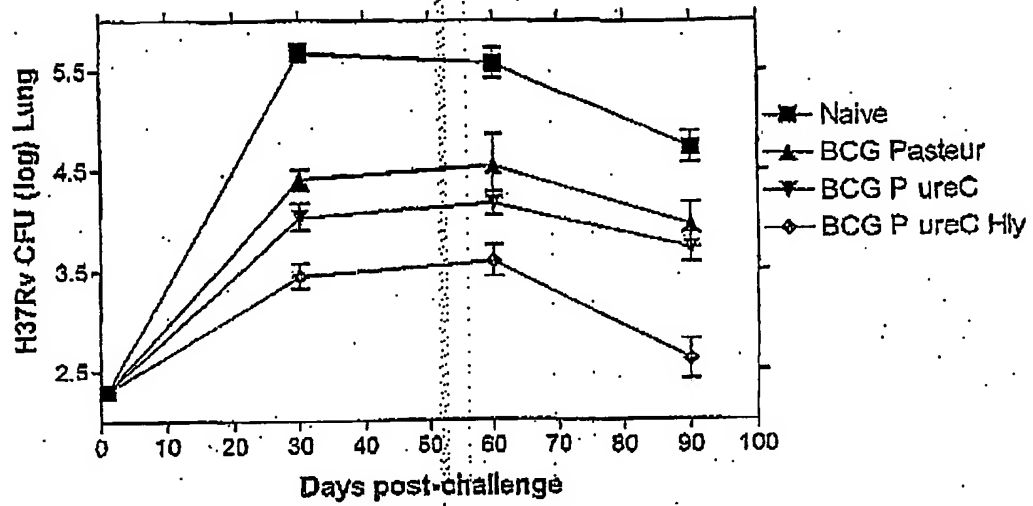
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Attorney Ref # 2009-01364-042303  
Prode, et al.

-1/1-

Figure 1



# SEQUENCE LISTING

## (1) GENERAL INFORMATION:

### (i) APPLICANT:

(A) NAME: Max-Planck-Gesellschaft zur Foerderung  
der  
Wissenschaften e.V.  
(B) STREET: Hofgartenstrasse 2  
(C) CITY: Muenchen  
(E) COUNTRY: Germany  
(F) POSTAL CODE (ZIP): 80539

(ii) TITLE OF INVENTION: Tuberculosis vaccine

(iii) NUMBER OF SEQUENCES: 2

### (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version  
#1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

### (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1881 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: linear

### (ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 1..1878

### (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

40	ATG ACA GAC GTG AGC CGA AAG ATT CGA GCT TGG GGA CGC CGA TTG ATG	48
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	1 5 10 15	
45	ATC GGC ACG GCA GCG GCT GTA GTC CTT CCG GGC CTG GTG GGG CTT GCC	96
	Ile Gly Thr Ala Ala Ala Val Val Leu Pro Gly Leu Val Gly Leu Ala	
	20 25 30	
50	GGC GGA GCG GCA ACC GCG GGC GCG TTC TCC CGG CCG GGG CTG CCG GTC	144
	Gly Gly Ala Ala Thr Ala Gly Ala Phe Ser Arg Pro Gly Leu Pro Val	
	35 40 45	
55	GAG TAC CTG CAG TCT GCA AAG CAA TCC GCT GCA AAT AAA TTG CAC TCA	192
	Glu Tyr Leu Gln Ser Ala Lys Gln Ser Ala Ala Asn Lys Leu His Ser	
	50 55 60	
60	GCA GGA CAA AGC ACG AAA GAT GCA TCT GCA TTC AAT AAA GAA AAT TCA	240
	Ala Gly Gln Ser Thr Lys Asp Ala Ser Ala Phe Asn Lys Glu Asn Ser	
	65 70 75 80	
60	ATT TCA TCC ATG GCA CCA CCA GCA TCT CCG CCT GCA AGT CCT AAG ACG	288
	Ile Ser Ser Met Ala Pro Pro Ala Ser Pro Pro Ala Ser Pro Lys Thr	
	85 90 95	

	CCA	ATC	GAA	AAG	AAA	CAC	GCG	GAT	GAA	ATC	GAT	AAG	TAT	ATA	CAA	GGA	336
	Pro	Ile	Glu	Lys	Lys	His	Ala	Asp	Glu	Ile	Asp	Lys	Tyr	Ile	Gln	Gly	
			100						105					110			
5	TTG	GAT	TAC	AAT	AAA	AAC	AAT	GTA	TTA	GTA	TAC	CAC	GGA	GAT	GCA	GTG	384
	Leu	Asp	Tyr	Asn	Lys	Asn	Asn	Val	Leu	Val	Tyr	His	Gly	Asp	Ala	Val	
			115					120					125				
10	ACA	AAT	GTG	CCG	CCA	AGA	AAA	GGT	TAC	AAA	GAT	GGA	AAT	GAA	TAT	ATT	432
	Thr	Asn	Val	Pro	Pro	Arg	Lys	Gly	Tyr	Lys	Asp	Gly	Asn	Glu	Tyr	Ile	
			130					135					140				
15	GTT	GTG	GAG	AAA	AAG	AAG	AAA	TCC	ATC	AAT	CAA	AAT	AAT	GCA	GAC	ATT	480
	Val	Val	Glu	Lys	Lys	Lys	Lys	Ser	Ile	Asn	Gln	Asn	Asn	Ala	Asp	Ile	
			145				150					155				160	
20	CAA	GTT	GTG	AAT	GCA	ATT	TCG	AGC	CTA	ACC	TAT	CCA	GGT	GCT	CTC	GTA	528
	Gln	Val	Val	Asn	Ala	Ile	Ser	Ser	Leu	Thr	Tyr	Pro	Gly	Ala	Leu	Val	
					165					170					175		
25	AAA	GCG	AAT	TCG	GAA	TTA	GTA	GAA	AAT	CAA	CCA	GAT	GTT	CTC	CCT	GTA	576
	Lys	Ala	Asn	Ser	Glu	Leu	Val	Glu	Asn	Gln	Pro	Asp	Val	Leu	Pro	Val	
					180				185					190			
30	AAA	CGT	GAT	TCA	TTA	ACA	CTC	AGC	ATT	GAT	TTG	CCA	GGT	ATG	ACT	AAT	624
	Lys	Arg	Asp	Ser	Leu	Thr	Leu	Ser	Ile	Asp	Leu	Pro	Gly	Met	Thr	Asn	
				195				200					205				
35	CAA	GAC	AAT	AAA	ATC	GTT	GTA	AAA	AAT	GCC	ACT	AAA	TCA	AAC	GTT	AAC	672
	Gln	Asp	Asn	Lys	Ile	Val	Val	Lys	Asn	Ala	Thr	Lys	Ser	Asn	Val	Asn	
			210				215					220					
40	AAC	GCA	GTA	AAT	ACA	TTA	GTG	GAA	AGA	TGG	AAT	GAA	AAA	TAT	GCT	CAA	720
	Asn	Ala	Val	Asn	Thr	Leu	Val	Glu	Arg	Trp	Asn	Glu	Lys	Tyr	Ala	Gln	
			225			230				235					240		
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	Ala	Tyr	Pro	Asn	Val	Ser	Ala	Lys	Ile	Asp	Tyr	Asp	Asp	Glu	Met	Ala	
					245					250					255		
50	TAC	AGT	GAA	TCA	CAA	TTA	ATT	GCG	AAA	TTT	GGT	ACA	GCA	TTT	AAA	GCT	816
	Tyr	Ser	Glu	Ser	Gln	Leu	Ile	Ala	Lys	Phe	Gly	Thr	Ala	Phe	Lys	Ala	
					260				265					270			
55	GTA	AAT	AAT	AGC	TTG	AAT	GTA	AAC	TTC	GGC	GCA	ATC	AGT	GAA	GGG	AAA	864
	Val	Asn	Asn	Ser	Leu	Asn	Val	Asn	Phe	Gly	Ala	Ile	Ser	Glu	Gly	Lys	
				275				280					285				
60	ATG	CAA	GAA	GAA	GTC	ATT	AGT	TTT	AAA	CAA	ATT	TAC	TAT	AAC	GTG	AAT	912
	Met	Gln	Glu	Glu	Val	Ile	Ser	Phe	Lys	Gln	Ile	Tyr	Tyr	Asn	Val	Asn	
			290				295					300					
65	GTT	AAT	GAA	CCT	ACA	AGA	CCT	TCC	AGA	TTT	TTC	GGC	AAA	GCT	GTT	ACT	960
	Val	Asn	Glu	Pro	Thr	Arg	Pro	Ser	Arg	Phe	Phe	Gly	Lys	Ala	Val	Thr	
			305			310					315				320		
70	AAA	GAG	CAG	TTG	CAA	GCG	CTT	GGA	GTG	AAT	GCA	GAA	AAT	CCT	CCT	GCA	1008
	Lys	Glu	Gln	Leu	Gln	Ala	Leu	Gly	Val	Asn	Ala	Glu	Asn	Pro	Pro	Ala	
					325					330					335		
75	TAT	ATC	TCA	AGT	GTG	GCG	TAT	GGC	CGT	CAA	GTT	TAT	TTG	AAA	TTA	TCA	1056
	Tyr	Ile	Ser	Ser	Val	Ala	Tyr	Gly	Arg	Gln	Val	Tyr	Leu	Lys	Leu	Ser	
					340				345					350			
80	ACT	AAT	TCC	CAT	AGT	ACT	AAA	GTA	AAA	GCT	GCT	TTT	GAT	GCT	GCC	GTA	1104
	Thr	Asn	Ser	His	Ser	Thr	Lys	Val	Lys	Ala	Ala	Phe	Asp	Ala	Ala	Val	
					355			360					365				
85	AGC	GGA	AAA	TCT	GTC	TCA	GGT	GAT	GTA	GAA	CTA	ACA	AAT	ATC	ATC	AAA	1152
	Ser	Gly	Lys	Ser	Val	Ser	Gly	Asp	Val	Glu	Leu	Thr	Asn	Ile	Ile	Lys	
				370			375						380				

	AAT TCT TCC TTC AAA GCC GTA ATT TAC GGA GGT TCC GCA AAA GAT GAA	1200
	Asn Ser Ser Phe Lys Ala Val Ile Tyr Gly Gly Ser Ala Lys Asp Glu	
	385 390 395 400	
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	405 410 415	
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	Ser Glu Tyr Ile Glu Thr Ser Lys Ala Tyr Thr Asp Gly Lys Ile	
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	Ile Tyr Leu Pro Gly Asn Ala Arg Asn Ile Asn Val Tyr Ala Lys Glu	
	515 520 525	
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	Cys Thr Gly Leu Ala Trp Glu Trp Trp Arg Thr Val Ile Asp Asp Arg	
	530 535 540	
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	Asn Leu Pro Leu Val Lys Asn Arg Asn Ile Ser Ile Trp Gly Thr Thr	
	545 550 555 560	
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	Leu Tyr Pro Lys Tyr Ser Asn Lys Val Asp Asn Pro Ile Glu Tyr Ala	
	565 570 575	
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	Leu Ala Tyr Gly Ser Gln Gly Asp Leu Asn Pro Leu Ile Asn Glu Ile	
	580 585 590	
65	AGC AAA ATC ATT TCA GCT GCA GTT CTT TCC TCT TTA ACA TCG AAG CTA	1824
	Ser Lys Ile Ile Ser Ala Ala Val Leu Ser Ser Leu Thr Ser Lys Leu	
	595 600 605	
70	CCT GCA GAG TTC GTT AGG CGC GGA TCC GGA ATT CGA AGC TTA TCG ATG	1872
	Pro Ala Glu Phe Val Arg Arg Gly Ser Gly Ile Arg Ser Leu Ser Met	
	610 615 620	
75	TCG ACG TAG	1881
	Ser Thr	
	625	

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 626 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Thr Asp Val Ser Arg Lys Ile Arg Ala Trp Gly Arg Arg Leu Met  
1 5 10 15  
Ile Gly Thr Ala Ala Ala Val Val Leu Pro Gly Leu Val Gly Leu Ala  
20 25 30  
Gly Gly Ala Ala Thr Ala Gly Ala Phe Ser Arg Pro Gly Leu Pro Val  
35 40 45  
Glu Tyr Leu Gln Ser Ala Lys Gln Ser Ala Ala Asn Lys Leu His Ser  
50 55 60  
Ala Gly Gln Ser Thr Lys Asp Ala Ser Ala Phe Asn Lys Glu Asn Ser  
65 70 75 80  
Ile Ser Ser Met Ala Pro Pro Ala Ser Pro Pro Ala Ser Pro Lys Thr  
85 90 95  
Pro Ile Glu Lys Lys His Ala Asp Glu Ile Asp Lys Tyr Ile Gln Gly  
100 105 110  
Leu Asp Tyr Asn Lys Asn Asn Val Leu Val Tyr His Gly Asp Ala Val  
115 120 125  
Thr Asn Val Pro Pro Arg Lys Gly Tyr Lys Asp Gly Asn Glu Tyr Ile  
130 135 140  
Val Val Glu Lys Lys Lys Lys Ser Ile Asn Gln Asn Asn Ala Asp Ile  
145 150 155 160  
Gln Val Val Asn Ala Ile Ser Ser Leu Thr Tyr Pro Gly Ala Leu Val  
165 170 175  
Lys Ala Asn Ser Glu Leu Val Glu Asn Gln Pro Asp Val Leu Pro Val  
180 185 190  
Lys Arg Asp Ser Leu Thr Leu Ser Ile Asp Leu Pro Gly Met Thr Asn  
195 200 205  
Gln Asp Asn Lys Ile Val Val Lys Asn Ala Thr Lys Ser Asn Val Asn  
210 215 220  
Asn Ala Val Asn Thr Leu Val Gln Arg Trp Asn Glu Lys Tyr Ala Gln  
225 230 235 240  
Ala Tyr Pro Asn Val Ser Ala Lys Ile Asp Tyr Asp Asp Glu Met Ala  
245 250 255  
Tyr Ser Glu Ser Gln Leu Ile Ala Lys Phe Gly Thr Ala Phe Lys Ala  
260 265 270  
Val Asn Asn Ser Leu Asn Val Asn Phe Gly Ala Ile Ser Glu Gly Lys  
275 280 285  
Met Gln Glu Glu Val Ile Ser Phe Lys Gln Ile Tyr Tyr Asn Val Asn  
290 295 300  
Val Asn Glu Pro Thr Arg Pro Ser Arg Phe Phe Gly Lys Ala Val Thr  
305 310 315 320  
Lys Glu Gln Leu Gln Ala Leu Gly Val Asn Ala Glu Asn Pro Pro Ala  
325 330 335

Tyr Ile Ser Ser Val Ala Tyr Gly Arg Gln Val Tyr Leu Lys Leu Ser  
 340 345 350  
 5 Thr Asn Ser His Ser Thr Lys Val Lys Ala Ala Phe Asp Ala Ala Val  
 355 360 365  
 Ser Gly Lys Ser Val Ser Gly Asp Val Glu Leu Thr Asn Ile Ile Lys  
 370 375 380  
 10 Asn Ser Ser Phe Lys Ala Val Ile Tyr Gly Gly Ser Ala Lys Asp Glu  
 385 390 395 400  
 Val Gln Ile Ile Asp Gly Asn Leu Gly Asp Leu Arg Asp Ile Leu Lys  
 405 410 415  
 15 Lys Gly Ala Thr Phe Asn Arg Glu Thr Pro Gly Val Pro Ile Ala Tyr  
 420 425 430  
 Thr Thr Asn Phe Leu Lys Asp Asn Glu Leu Ala Val Ile Lys Asn Asn  
 435 440 445  
 20 Ser Glu Tyr Ile Glu Thr Thr Ser Lys Ala Tyr Thr Asp Gly Lys Ile  
 450 455 460  
 25 Asn Ile Asp His Ser Gly Gly Tyr Val Ala Gln Phe Asn Ile Ser Trp  
 465 470 475 480  
 Asp Glu Val Asn Tyr Asp Pro Glu Gly Asn Glu Ile Val Gln His Lys  
 485 490 495  
 30 Asn Trp Ser Glu Asn Asn Lys Ser Lys Leu Ala His Phe Thr Ser Ser  
 500 505 510  
 Ile Tyr Leu Pro Gly Asn Ala Arg Asn Ile Asn Val Tyr Ala Lys Glu  
 515 520 525  
 35 Cys Thr Gly Leu Ala Trp Glu Trp Trp Arg Thr Val Ile Asp Asp Arg  
 530 535 540  
 40 Asn Leu Pro Leu Val Lys Asn Arg Asn Ile Ser Ile Trp Gly Thr Thr  
 545 550 555 560  
 Leu Tyr Pro Lys Tyr Ser Asn Lys Val Asp Asn Pro Ile Glu Tyr Ala  
 565 570 575  
 45 Leu Ala Tyr Gly Ser Gln Gly Asp Leu Asn Pro Leu Ile Asn Glu Ile  
 580 585 590  
 50 Ser Lys Ile Ile Ser Ala Ala Val Leu Ser Ser Leu Thr Ser Lys Leu  
 595 600 605  
 Pro Ala Glu Phe Val Arg Arg Gly Ser Gly Ile Arg Ser Leu Ser Met  
 610 615 620  
 55 Ser Thr  
 625

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